THE JOURNAL OF INFECTIOUS DISEASES • VOL. 156, NO. 6 • DECEMBER 1987

Isolation of a Hantavirus from a Severely Ill Patient with Hemorrhagic Fever with Renal Syndrome in Greece

Attention has recently been drawn to a severe form of hemorrhagic fever with renal syndrome (HFRS) found in the Balkan region of Europe [1-4]. This disease is characterized by fever, abdominal or back pain, conjunctival injection, renal insufficiency, and a significant mortality rate (currently estimated to be ~14% in Greece [5]). The disease resembles Korean hemorrhagic fever (KHF) of Korea and epidemic hemorrhagic fever (EHF) of China, rather than the milder nephropathia epidemica (NE) of Scandinavia and Western Europe [5, 6]. Patients who have recovered from this disease possess antibodies that react to highest titers with Hantaan virus, the cause of KHF and EHF, rather than with Puumala or Seoul viruses, the other known causes of HFRS [4, 5]. We have previously suggested [4], based on serological and epidemiological studies, that the disease found in Greece may be caused by a virus closely related or identical to the prototype Hantaan virus. In this communication, we report the isolation of a hantavirus from the urine of a severely ill patient with HFRS who was infected in northern Greece and the preliminary serological evidence to suggest that this virus represents a unique strain of hantavirus that is closely related to the prototype Hantaan virus.

Patient and Methods

Patient. A 21-year-old male soldier with a history of chronic peptic ulcer was admitted to the General Military Hospital of Thessaloniki on 28 March 1986 with a three-day history of fever (39 C), nausea, vomiting, diarrhea, weakness, myalgia, backache, and abdominal pain. He had been assigned to a military post near the village of Porogia in northern Greece before the onset of disease. On physical examination there were conjunctivitis, edematous eyelids, facial flushing, and "coffee-ground" vomit. The patient's heart and lungs were normal. The abdomen was diffusely sensitive to palpatation but was not rigid. The patient's arterial blood pressure was 40/30 mm Hg, his pulse was 100 per minute, and his central venous pressure was 5 cm of H_2O. Laboratory evaluation done at admission showed a hematocrit level of 59% and a white blood cell count of 18,770/mm³, with 60% segmented neutrophils, 16% small lymphocytes, 10% large lymphocytes, 8% monocytes, and 6% eosinophils. Nasogastric suction produced 300 ml of turbid, brownish liquid; a urinary catheter drained 120 ml of blood-stained urine during the first 24 hr of hospitalization.

Over the next 48 hr, the patient's clinical condition deteriorated, and he developed pulmonary edema and skin, nasal, and gastrointestinal hemorrhages. Gastroscopy revealed ulceration of the lower third of the esophagus and diffuse hemorrhagic gastritis. Because of progressive renal failure and the clinical evidence of disseminated intravascular coagulation, the patient was transferred to the renal unit of the American Hellenic Educational Progressive Association Hospital (Thessaloniki) on 31 March 1986. Renal hemodialysis was begun via an external shunt; this procedure led to an initial clinical improvement. On the second day in the renal unit (seventh day after onset), however, the patient's clinical picture worsened because of recurrent and persistent pulmonary edema. The patient was then transferred to the intensive care unit, where he received artificial ventilation. Hemodialysis was continued for 14 hr, with removal of 7 liters of fluids. Supportive treatment included administration of theophylline, hydrocortisone, sodium bicarbonate, digoxin, and calcium gluconate. The patient gradually improved with daily hemodialysis and respiratory support. He remained in intensive care for 20 days, then was transferred to a general medicine ward for convalescence. At that time the patient's urine output was 2,500 ml/24 hr, his creatinine level was 2.1 mg/100 ml, and his level of blood urea nitrogen was 87 mg/100 ml. On 24 April 1986 (31st day after onset), the patient was discharged. Six months later, he exhibited no clinical sequelae, and laboratory test results had returned to normal.

Serum drawn on the seventh and 13th days after the onset of disease had IgG antibody titers of 512 and 4,096, respectively, when tested by indirect immunofluorescent antibody (IFA) assay for Hantaan virus. The IgM titer by IFA on day 7 after onset was 1,024.

Isolation of virus. Both whole blood and urine were obtained on the seventh day of illness, when the patient improved slightly after the initial dialysis treatment. One-milliliter aliquots of blood were inoculated onto Vero E-6 (ATCC CRL 1586) cells grown in 25-cm² plastic flasks. Inoculated flasks were incubated at 37 C for 15 days, then the cells were suspended with trypsin and passed to fresh flasks. While suspended, some cells were used to prepare 10-well spot slides, which were then fixed in cold acetone

Received for publication 26 June 1987, and in revised form 3 August 1987.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

This work was supported by grant DAMD 17-87-G-7019 from the U.S. Army Medical Research and Development Command.

We thank Drs. C. J. Peters and G. A. Saviolakis for editorial assistance and Aleka Varna for laboratory support.

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and examined for characteristic hantavirus antigen by IFA assays with human antibody to Hantaan virus. A total of 6 ml of urine was collected from the patient's catheter and immediately alkalized with sodium bicarbonate. Aliquots of 0.25, 0.5, 1.0, and 1.5 ml of urine were inoculated into separate 25-cm² flasks of Vero E-6 cells in the presence of 8 ml of growth medium. Inoculated flasks were incubated for 15 days at 37 C, then cells were suspended, passed to fresh flasks, and 10-well spot slides prepared and examined for characteristic hantavirus cytoplasmic fluorescence by using reference antibodies to Hantaan virus. When nearly 100% of the inoculated cells expressed characteristic cytoplasmic fluorescence by IFA, supernatant culture fluids were titrated by plaque assay on Vero E-6 cells, and plaque-reduction neutralization (PRN) tests were performed as described below. Isolation attempts were considered negative if characteristic hantavirus antigen was not detected by IFA after 50 days of serial, blind passage.

Characterization of virus. Virus isolated from urine was characterized by cross-IFA and -PRN tests and for reactivity with reference immune sera and monoclonal antibodies produced to prototype Hantaan virus, strain 76-118. Immunofluorescent-antibody assays used Vero E-6 cells that had been infected with various hantaviruses, fixed to 10-well spot slides, and stored at −20 C before use. Reference or test sera were examined at dilutions of 1:8-≥1:2,048 in fourfold increments; sera were considered positive if characteristic cytoplasmic fluorescence was observed. Reference control sera included convalescent serum from a patient with KHF who was infected in Korea, convalescent serum from a patient with NE who was infected in Sweden, convalescent sera from several Greek patients with HFRS who had previously been diagnosed in our laboratories, and sera obtained from laboratory rats experimentally infected with Seoul virus. Viruses tested included the prototype strain of Hantaan virus, strain 76-118; Puumala virus, strain 83-223L; and Seoul virus, strain 80/30. PRN tests were done on Vero E-6 cells, according to procedures described previously [6], by using the same viruses and antisera described above. Titer was recorded as the reciprocal of the highest serum dilution reducing >50% of the plaque dose. Spot slides of Vero E-6 cells infected with the human isolate from Greece were tested by IFA with several monoclonal antibodies produced to Hantaan virus strain 76-118.

Results

Hantavirus-specific antigen was found only in cells inoculated with 0.5 ml of our patient's urine. At 15 days after inoculation, ~5% of the cells exhibited characteristic cytoplasmic fluorescence when tested by IFA. On subsequent passage, the percentage of cells expressing antigen rose to 20%-25% at the second passage and to virtually 100% by the fifth passage. Spot slides prepared with cells infected with the isolate were then tested with immune sera or with monoclonal antibodies to Hantaan virus or other hantaviruses. As shown in table 1, high titers were seen by IFA when human immune sera were tested with either the Greek isolate or the prototype Hantaan virus. Similar, although not identical, reactivity was noted when Hantaan virus and the Greek isolate were tested with six monoclonal antibodies to Hantaan virus.

The isolate was next compared with several other hantaviruses in cross-PRN tests. Results of these comparisons are shown in table 2. The Greek isolate reacted to equivalent titers with both the patient's immune serum and immune sera from rats experimentally infected with Seoul virus; the isolate reacted to lower titers with immune serum from a patient with KHF. Prototype Hantaan virus reacted to only a twofold higher titer when tested with the convalescent serum from a patient with KHF as compared with our patient's convalescent serum and to substantially lower titer with the rat antiserum to Seoul virus. Neither virus reacted to significant titers to immune serum from a patient with NE, nor did antibody to these viruses react to high titer with Puumala virus.

Both the Greek isolate and the prototype Hantaan virus were next tested, by PRN tests, with convalescent sera from a number of Greek patients with severe HFRS who had been diagnosed in our laboratories over the past five years. Results of these tests are also shown in table 2. In most cases, the highest titers were found with the Greek isolate rather than with Hantaan virus.

We conclude from these results that the virus recovered from this critically ill patient was a hantavirus that was closely related to prototype Hantaan virus. Sufficient serological distinction was found to suggest that the virus isolated represents a unique strain among the hantaviruses.

Table 1. Antibody titers, by IFA, to Hantaan virus and to the Greek isolate.

Sera	Antibody titers to Hantaan virus	Antibody titers to Greek isolate
Immune sera from patient with KHF	8,192	4,096
Immune sera from Greek patient		
Day 7	512	NT*
Day 13	4,096	8,192
Monoclonal antibodies to Hantaan virus		
HC02-BH11	>2,048	>2,048
BD01-BB08	>2,048	>2,048
EC02-BG01	>2,048	>2,048
JD04-AC06	512	>2,048
FD03-AA11	512	512
GD05-BB09	32	512

* NT, not tested.

Table 2. PRN tests with Hantaan virus, Seoul virus, Puumala virus, and the virus from our patient with HFRS, and a comparison of antibody titers to Hantaan virus and the Greek isolate with sera from previously diagnosed Greek patients with HFRS.

Sera	Antibody titers to			
	Hantaan virus	Seoul virus	Puumala virus	Greek isolate
Immune sera from patient with KHF	**2,048**	<8	64	256
Rat antisera to Seoul virus	256	**2,048**	<16	1,024
Immune sera from patient with NE	<8	<8	**1,024**	16
Sera from patient studied				
Day 14	1,024	<8	32	**1,024**
Day 24	32	<8	<8	**512**
Sera from previously diagnosed patients				
Patient 3				
Day 8	64	NT	NT	512
Day 106	128	NT	NT	2,048
Patient 8				
Day 14	1,024	NT	NT	2,048
Day 24	32	NT	NT	512
Patient 13				
Day 20	128	NT	NT	2,048
Day 35	256	NT	NT	512
Patient 15, day 28	256	NT	NT	256
Patient 18, day 10	1,024	NT	NT	2,048

NOTE. Data are the reciprocal of the highest dilution neutralizing 50% of the plaque dose (~100 pfu). NT, not tested.

We propose the name *Porogia virus* for this strain of hantavirus, after the location in Greece where this patient was likely to have been infected.

Discussion

This is the first detailed case history of the severe form of HFRS as it occurs in Greece. Although distinct phases that have been identified in Asian patients with HFRS were not recognized as such in this case history, it is clear that this patient suffered a course of illness virtually identical to that seen with severely ill patients in Asia [7, 8]. The acute onset of febrile illness was followed by hypotension, oliguria, and hemorrhage, with diuresis heralding improvement, albeit with a prolonged convalescence. At admission, the patient was already hypotensive and in shock. The hemorrhagic manifestations seen, which continued into the oliguric phase, were consistent with previous reports from Asian patients. The initial presentation was confusing because the patient had a history of chronic peptic ulcer disease, but endoscopy established esophageal ulceration and diffuse gastritis as sites of hemorrhage.

It is apparent that the virus recovered from the patient was responsible for the disease seen. The patient developed rising antibody titers to the isolated agent, and the clinical illness was consistent with that typically associated with hantavirus infections. No other hantaviruses were being handled in the laboratory at the time of the isolation, a situation ruling out the chance that the isolate was a laboratory contaminant.

Serological comparison of the isolated virus to other hantaviruses establishes it as a member of this group. Although the IFA test results revealed little difference between the isolate and Hantaan virus, the more specific PRN tests demonstrated sufficient differences to suggest that the isolated virus represents a unique strain of hantavirus; however, additional genetic analyses are required to establish the precise relationship of the isolate to the prototype Hantaan virus [9].

A comparison of PRN titers to both Hantaan virus and the Greek isolate with titers of convalescent sera from patients previously diagnosed with severe HFRS found highest titers to the Greek virus, a result suggesting that this virus may have been responsible for many of these cases [1, 3–5].

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